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博 士 学 位 论 文

人血清转铁蛋白-顺铂的质谱与波谱特性及其靶向诱导肝癌细胞凋亡的分子机制研究

The Human Serum Transferrin-Cisplatin's Characteristics of Mass Spectrometry and Spectrum and the Molecular Mechanism of Targeting and Apoptosis to Hepatoma Cell from the Complex

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厦门大学博士论文摘要库

中文摘要

外科治疗,放射性治疗(放疗),化学药物治疗(化疗)、和免疫(生物)治疗是目前癌症治疗的四大主要手段,其中化疗法具有能进行全身性癌症治疗、多发性癌症治疗、以及癌症根治率相对较高等优点,成为应用最广泛、发展最快的治疗手段。但是,传统的化疗药物都不能特异性的杀伤癌细胞,化疗效果和严重毒副作用并存。疗效依赖于药物毒性、给药剂量、给药途径、给药次数和疗程的各种化疗药物均具有细胞毒性极强、给药剂量大,并易产生耐药性等缺陷,给化疗患者带来难以忍受的痛苦。近十年来,高效靶向化疗成为肿瘤治疗学的研究热点之一,在组织器官水平、细胞水平和分子水平上的靶向治疗都有大量的研究报道,部分研究成果已应用于临床治疗。

本文通过小批量制备人血清转铁蛋白(Human serum transferrin, HTF),通过质谱与波谱特性研究了 HTF 络合顺铂(Cis-diamminedichloroplatinum, cisplatin/CDDP)能力,构建了 HTF-CDDP 运输载体,并在体外考察了 HTF-CDDP 细胞水平的靶向能力,在昆明小鼠体内考察了 HTF-CDDP 组织器官水平的靶向能力。通过筛选与鉴定 HTF-CDDP 诱导肿瘤细胞凋亡的差异蛋白质,深入了解 HTF-CDDP 诱导肿瘤细胞以及肿瘤细胞对 CDDP 产生耐药性的分子机理,为 HTF-CDDP 在分子水平靶向凋亡肿瘤细胞的后续研究奠定基础,同时也为传统化疗药物治疗肿瘤疾病提供新思路、新途径和新颖分析技术,立题研究具有重要的科学意义,和潜在应用价值,主要研究内容如下:

第一: HTF 制备及异构体分析

采用线性梯度和均匀天然聚丙烯酰胺凝胶电泳联用的两次电泳技术小批量制备 HTF。选用反相液相色谱法(Reverse phase high performance liquid chromatography, RP-HPLC)、阴离子交换色谱法、等电聚焦聚丙烯酰胺凝胶电泳法(Isoelectric focusing polyacrylamide gel electrophoresis, IEF-PAGE)、基质辅助激光解析电离飞行时间质谱技术(Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, MALDI-TOF-MS)、肽质量指纹图谱技术(Peptide mass fingerprinting, PMF)等进行 HTF 及异构体组成分析。结果表明 RP-HPLC 和 IEF-PMF 联用技术是最有效的 HTF 异构体组成分析方法,发现 HTF

由 asialotransferrin、monosialotransferrin、disialotransferrin、trisialotransferrin、tetrasialotransferrin、pentasialotransferrin、hexasialotransferrin、septimsialotransferrin 等 8 个糖链缺失异构体, 和 HTF-Dchi、HTf-C1、HTf-B2 等三个遗传型异构体组成, 其中 HTF-C1 型是主要遗传型异构体, 占有遗传型异构体的 95%以上, 而带 4 个糖链分支的 tetrasialotransferrin 异构体占 HTF 总量的 80%左右, 是糖链缺失 HTF 的主要异构体。

第二: HTF 络合 CDDP 能力的研究

选用圆二色谱(Circular dichroism spectrum, CD)、荧光光谱 (Fluorescence spectrum, FS)、红外光谱(Infrared spectrum, IR)、电感耦合等离子体质谱 (Inductively coupled plasma mass spectrometry, ICP-MS) 等分析方法研究不同 pH 值条件下, HTF 结构转换趋势和络合 CDDP 能力。在酸性条件下, 反应体系的 pH 值越低, HTF 的结构越松散; 当 pH<3.5 时, HTF 分子结构会发生不可逆的变化, 但在 pH3.5-7.25 范围内, 其分子结构随 pH 的变化呈现不同的开放程度。三氟乙酸 (Trifluoroacetic acid, TFA) 能改善 HTF 络合 CDDP 的能力, 其络合 CDDP 数目依赖于反应体系的 pH 值。建立 pH 影响 HTF 络合 CDDP 能力的反应模型, 构建 HTF-CDDP 复合物, 其络合 CDDP 数量可高达 10 CDDP/HTF。

第三: HTF-CDDP 靶向 HepG2 肿瘤细胞能力及其诱导凋亡的分子机制的研究

通过 FITC 标记 HTF-CDDP 靶向运输载体, 激光扫描共聚焦显微镜技术分析 HTF-CDDP 靶向及胞饮进入肿瘤细胞的能力, ICP-MS 技术分析 HTF-CDDP 在细胞内外释放 CDDP 的情况, 流式细胞术分析 HTF-CDDP 靶向肿瘤细胞引起肿瘤凋亡的效果, 蛋白质组学及 Realtime-PCR 技术分析 CDDP 与 HTF-CDDP 凋亡 HepG2 细胞的分子机制差异性途径, 研究 CDDP 和给药途径对 HepG 2 肝癌耐药性产生的影响。结果表明, HTF-CDDP 能够有效的靶向肿瘤细胞, 并与 holo-HTF 竞争性地与 TFR 识别与结合, 通过胞饮途径进入细胞内释放 CDDP, 导致肿瘤细胞凋亡, 说明 HTF-CDDP 靶向运输载体能在细胞水平上有效的靶向凋亡肿瘤细胞。Apo-HTF-CDDP 和 CDDP 诱发肿瘤细胞凋亡的分子机制存在差异, 主要表现在对 ATM/ATR、P73、Akt、BAD、P27、mTOR、GADD45A、53BP1、PCNA 和 Rad 51 等关键蛋白/激酶的影响上。同时发现 ATM、ATR、Akt 等蛋白

质是细胞应对 CDDP 引起 DNA 损伤后的重要反应蛋白，如何通过抑制这些蛋白的功能，来提高 CDDP 等 DNA 毒性化疗药物的抗癌疗效值得深入研究。

第四：TF-CDDP 在昆明小鼠体内分布情况

构建昆明小鼠血清转铁蛋白 (Mouse serum transferrin, MSTF) -CDDP，并研究 MSTF-CDDP 和 CDDP 在昆明小鼠各器官中的分布规律与代谢趋势。在给药后 1、3、8、16、24、72 h 分离昆明小鼠血清、血细胞、肝脏、肾脏、脾脏、肺、心、脑、脊髓和肿瘤等组织，浓硝酸充分消解后用 ICP-MS 检测各组织的 CDDP 浓度，分析 CDDP 和 MSTF-CDDP 在昆明小鼠体内分布与代谢情况，同时以组织切片技术和透射电镜技术分析 CDDP 和 MSTF-CDDP 处理下的肾脏损伤情况。结果表明，pH 调节血清转铁蛋白络合 CDDP 数量的反应模型同样适合于 MSTF 与 CDDP 的络合，成功构建了络合 10 个左右 CDDP 分子的 MSTF-CDDP 靶向运输载体；MSTF-CDDP 能够通过内吞小泡的形式进入细胞中，表明 MSTF-CDDP 能够有效的与细胞表面受体结合，并通过 TF-TFR 胞饮传递系统进入细胞内，进一步证明 MSTF-CDDP 靶向运输的可能性；MSTF-CDDP 能减少 CDDP 在血细胞中的滞留，是有效提高 CDDP 药效和降低血液毒性的途径之一；MSTF-CDDP 在昆明小鼠体内能够富集于肿瘤组织及大部分内脏器官中，并在内脏器官、肿瘤组织内维持更长时间的高药物浓度，尤其是在肝脏和脾脏组织中，但没有表现出在脑部和脊髓中的富集，不会增强其神经毒性；同时 MSTF-CDDP 也没有明显的肾脏毒性提高的表现，表明 MSTF-CDDP 能够在相对低血药浓度（低毒副作用）条件下，在组织器官水平靶向肝脏、脾脏等器官，并在细胞水平靶向肿瘤细胞，对靶向治疗肝脏、脾脏癌症具有巨大潜力。

关键词：肝癌，靶向治疗，细胞凋亡，转铁蛋白，顺铂，蛋白质组学

Abstract

Four therapies including surgery, radiotherapy, chemotherapy, and immunotherapy are the major clinical therapies of cancer. In which chemotherapy is most widely used due to its potential of treating with systemic cancers and recurrent cancers, as well as its relatively high cure rate. However, few traditional drugs for chemotherapy can selectively kill cancer cells, which means a lot of normal cells will be killed during chemotherapy. Furthermore, chemotherapy drugs, effect of which depends on drug toxicity, dosage, time and route of administration, often bring unbearable pains to patients because of their high cytotoxicity and potentials of induce surviving cells with drug resistance. Over the past decade, chemotherapies based on targeted drug delivery systems have been one of the focused stems of cancer therapy, and a lot of research results have been reported about chemotherapy drugs targeting tissues/organs, cells and molecules, some of which have been used in clinical therapy of cancer.

Here we prepared a small quantity of human serum transferrin (HTF), and studied on the binding ability of HTF with cisplatin based on the HTF-CDDP characteristics of both mass spectrometry and spectrum, and investigated the targeting ability of HTF-CDDP to cancer cells in vitro, and to organs/ tissues in vivo. In addition, we detected and identified the differently regulated proteins in the HepG 2 cells that were induced to apoptosis by CDDP and HTF-CDDP, and further investigated the molecular mechanism of apoptosis and drug resistance of them. We suppose the projects have important scientific significance and potential of clinical therapy. The main contents are as follows:

1. Preparation of HTF and analysis of its isforms

A combined electrophoresis method of both the linear gradient natural polyacrylamide gel electrophoresis (PAGE_N) and uniform natural PAGE_N is employed to prepare a small quantity of HTf from human serum. Reverse phase high performance liquid chromatography (RP-HPLC), anion exchange chromatography,

matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, isoelectric focusing (IEF), and peptide mass fingerprinting (PMF) techniques are then used to detect the isoforms of HTf.

Results show that the combined a combined method of IEF-PMF and RP-HPLC is perfectly suitable for detection of the isoforms of HTf. By which It 8 carbohydrate-deficient transferrin (CDT) variants defined as asialotransferrin, monosialotransferrin, disialotransferrin, trisialotransferrin, tetrasialotransferrin, pentasialotransferrin, hexasialotransferrin, septimsialotransferrin, respectively, and 3 genetic variants as HTF-Dchi, HTf-C1, HTf-B2, respectively, are detected. Furthermore, results also show that HTF-C1 account for ~95% of all genetic variants of HTF, and tetrasialotransferrin account for ~ 80% of all glycosylation variants of HTF.

2. Studying on the binding ability of HTF with cisplatin

Circular dichroism spectrum (CD), fluorescence spectrum (FS), infrared spectrum (IR), and ICP-MS spectrum were employed to analysis of both conformational change and binding ability of HTF with CDDP. Results showed that lower pH value of acidic solution will induce more loose conformation of HTF, and an irreversible conformational change will occur to HTF when pH value of solution lower than 3.5. However, how much the HTF conformation opening was found depended on the pH value between pH3.5-7.25. Results also indicated that trifluoroacetic acid (TFA) can improve the binding ability of HTF to CDDP, based on which a model of HTF binding with and removing the CDDP adjusted by pH value using TFA was established, and a complex of HTF binding with 10 molecular CDDP each was prepared.

3. Studying on the targetting ability of HTF-CDDP to HepG 2 cells and the molecular mechanism of apoptosis of HepG 2 cells induced by the complex

Drug delivery vector of HTF-CDDP was labeled with fluorescein isothiocyanate (FITC), and Laser Scanning Confocal Microscopy (LSCM) was employed to analysis of the vector ability of targeting and endocytosis into tumor cells. ICP-MS spectrum was employed to analysis the CDDP release from HTF-CDDP vector both inside and

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